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Phenotype-associated inbreeding biases estimates of inbreeding depression in a wild bird population

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Abstract

Inbreeding depression is usually quantified by regressing individual phenotypic values on inbreeding coefficients, implicitly assuming there is no correlation between an individual's phenotype and the kinship coefficient to its mate. If such an association between parental phenotype and parental kinship exists, and if the trait of interest is heritable, estimates of inbreeding depression can be biased. Here we first derive the expected bias as a function of the covariance between mean parental breeding value and parental kinship. Subsequently, we use simulated data to confirm the existence of this bias, and show that it can be accounted for in a quantitative genetic animal model. Finally, we use long-term individual-based data for white-throated dippers (*Cinclus cinclus*), a bird species in which inbreeding is relatively common, to obtain an empirical estimate of this bias. We show that during part of the study period, parents of inbred birds had shorter wings than those of outbred birds, and as wing length is heritable, inbred individuals were smaller, independent of any inbreeding effects. This resulted in the overestimation of inbreeding effects. Similarly, during a period when parents of inbred birds had longer wings, we found that inbreeding effects were underestimated. We discuss how such associations may have arisen in this system, and why they are likely to occur in others, too. Overall, we demonstrate how less biased estimates of inbreeding depression can be obtained within a quantitative genetic framework, and suggest that inbreeding and additive genetic effects should be accounted for simultaneously whenever possible.

Introduction

In spatially fragmented environments, especially if populations are small and isolated, matings among relatives (i.e. inbreeding) are often common (Lande, 1988; Keller, 1998). Inbreeding increases homozygosity, and this, in turn, leads to (i) (partially) deleterious recessive alleles being expressed more often, (ii) a reduction in the frequency of the fittest genotype at loci showing overdominance and/or (iii) changes in gene interactions, all of which may negatively affect trait values and fitness (Crow & Kimura, 1970, p 78–80). Since

Darwin (1876), numerous studies testing for negative consequences of inbreeding (i.e. inbreeding depression) have shown that inbreeding depression is common, both in captive and in wild populations (Charlesworth & Charlesworth, 1987; Keller & Waller, 2002). In addition to individual-level effects on a variety of traits such as body mass, survival and fecundity in a range of plant and animal species, including humans, (e.g. DeRose & Roff, 1999; Richards, 2000; Kruuk *et al.*, 2002; Szulkin *et al.*, 2007; Postma *et al.*, 2010; Hemmings *et al.*, 2012) (but see Duarte *et al.*, 2003; Thünken *et al.*, 2007), the fitness-related consequences of inbreeding may also have population-level consequences, threatening the persistence of small populations (Newman & Pilson, 1997; Nieminen *et al.*, 2001).

The degree of inbreeding of an individual is measured as its coefficient of inbreeding, that is the probability of two alleles being identical by descent (IBD) (Wright's

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inbreeding coefficient f ; Wright, 1922; Malécot, 1948). Inbreeding coefficients can be obtained from pedigree data, in which case they are estimated relative to a base population consisting of presumed unrelated founders and immigrants. Alternatively, inbreeding coefficients can be inferred from multilocus genotype data (see Balloux *et al.*, 2004; Slate *et al.*, 2004; and Bérénos *et al.*, 2014 for evaluations of marker-based inbreeding estimates). Having a (pedigree- or marker-based) measure of inbreeding for each individual, we can test for inbreeding depression by regressing phenotypic values on individual inbreeding coefficients in a linear (mixed) model framework. Typically, larger trait values are associated with higher fitness (e.g. body mass, number of offspring, survival probability), and hence, studies testing for inbreeding effects typically find that inbred individuals have smaller trait values than outbred ones (with few exceptions like laying date in birds, where smaller values are typically associated with higher fitness; Gienapp *et al.*, 2006; Keller *et al.*, 2006). Although the term inbreeding depression is sometimes reserved for fitness-related traits, here we use it to describe any relationship between inbreeding and phenotype, irrespective of the relationship of this trait with fitness.

Inferring inbreeding depression from the relationship between phenotype and inbreeding coefficient assumes that inbreeding individuals are a random subsample of the population with respect to the trait of interest. For example, it assumes that there is no correlation between an individual's phenotype and the kinship coefficient to its mate, that is the inbreeding coefficient of its offspring. However, if, for example, related mates are characterized by lower trait values than unrelated ones, inbred offspring will have parents with lower trait values. If the trait is heritable, inbred offspring will be characterized by small trait values not only due to the potential effects of inbreeding, but also due to the additive genetic effects passed on by their parents. Hence, if additive genetic effects are not accounted for, this scenario will result in an overestimation of the magnitude of inbreeding depression. Likewise, the magnitude of inbreeding depression may be underestimated if inbred offspring have parents with higher trait values.

Lynch & Walsh (1998, p. 270–272) highlighted this issue, and Reid *et al.* (2008) showed that associations between parental phenotypes and parental relatedness may occur in natural populations. Although Uimari & Kennedy (1990) quantified the bias in estimates of inbreeding depression introduced by ignoring additive genetic effects using simulations, we are not aware of empirical studies that have directly quantified the bias in estimates of inbreeding depression caused by phenotype-associated inbreeding, despite the fact that there are reasons to believe that this may be a reasonably widespread phenomenon. For example, in an island population of song sparrows (*Melospiza melodia*), males with specific phenotypes (such as earlier hatching date,

shorter tarsi and lower survival probability) paired with close relatives more often than expected by chance (Reid *et al.*, 2008). Similarly, in collared flycatchers (*Ficedula albicollis*), offspring born late in the season are more often inbred than those born earlier in the season (Kruuk *et al.*, 2002), and in Seychelles warblers (*Acrocephalus sechellensis*), subordinate females mate with relatives more often than expected by chance (Richardson *et al.*, 2004).

Phenotype-associated inbreeding can arise for different reasons (see also Reid *et al.*, 2008). For example, individuals of higher quality might be able to avoid inbreeding more effectively than individuals of lower quality (e.g. Richardson *et al.*, 2004). Alternatively, given high philopatry and random mate choice, individuals with many siblings (and therefore a higher breeding value for fecundity) have a higher probability of pairing with a sib (Van Noordwijk & Scharloo, 1981). Furthermore, phenotype-associated inbreeding can be generated by phenotype-dependent dispersal, as dispersers typically have lower probabilities of inbreeding than philopatric individuals (e.g. Szulkin & Sheldon, 2008) and differ phenotypically from the latter. For example, in birds and many insects, dispersal behaviour is a function of body size, especially wing length, with bigger- or longer-winged individuals dispersing further (Paradis *et al.*, 1998; Skjelseth *et al.*, 2007; Dawideit *et al.*, 2009) (but see Chaput-Bardy *et al.*, 2010). In line with this, differences between philopatric individuals and dispersers have been detected in a range of morphological, behavioural and life-history traits, and in taxa ranging from single-cell species to primates (reviewed in Ronce & Clobert, 2012). In summary, estimates of inbreeding depression may be biased by phenotype-associated inbreeding under fairly general conditions.

Here we provide a quantitative investigation of the degree to which phenotype-associated inbreeding may bias estimates of inbreeding depression. To this end, we first use quantitative genetic theory to derive the expected bias in estimates of inbreeding depression due to phenotype-associated inbreeding for a trait with an additive genetic basis. Subsequently, we use simulated data to confirm the existence of this bias, and show that by using a quantitative genetic animal model, which explicitly accounts for additive genetic differences between inbred and outbred offspring, we are able to obtain more accurate estimates of inbreeding depression. Finally, we provide an empirical test of the effect of phenotype-associated inbreeding, using a long-term individual-based data set for white-throated dippers (*Cinclus cinclus*). We take wing length, which in this species is associated with dispersal behaviour, as our trait of interest and ask whether the kinship coefficient of mates is correlated with their wing length and whether such a correlation results in a biased estimate of inbreeding effects.

Materials and methods

Theory and simulations

Using standard quantitative genetic theory (Falconer & Mackay, 1996), we derive the effect of phenotype-associated inbreeding on estimates of the effect of inbreeding. To this end, we write an individual's phenotype (y) as a function of the population mean (μ), its breeding value (a), an inbreeding effect (b) and a residual environmental deviation (e). We show how the covariance between phenotype and inbreeding coefficient ($\sigma(y, f)$), is related to the covariance between mean parental breeding value and parental kinship ($\sigma(a, \theta)$), and how the latter is related to the covariance between mean parental phenotype and parental kinship ($\sigma(y, \theta)$), a quantity that can readily be observed.

We then compare these findings to the results from simulated data, which are analysed using both linear regression and a quantitative genetic animal model (Henderson, 1950; Kruuk, 2004). In short, we simulated phenotypes for three generations of individuals. In a first step, a population of 1000 females and 1000 males was created, all of them being unrelated (generation P). Phenotypes were simulated as the sum of a normally distributed breeding value (a) and residual environmental value (e), both with a mean of 0 and with variances equal to the additive genetic variance (V_A) and the environmental variance (V_E), respectively. The population mean (μ) was set to 0. Because $V_P=1$ in all our simulations, V_A (or $\sigma^2(a)$) was equal to the heritability h^2 , and V_E (or $\sigma^2(e)$) was equal to $1-h^2$. Following random mating, each pair subsequently produced one female and one male offspring (generation F1). Again, phenotypes were the sum of a breeding value and a random residual environmental value, and the breeding value equalled the sum of the mean of the parental breeding values and a random Mendelian sampling term. The latter was drawn from a normal distribution with mean zero and variance $\sigma^2 = 0.5 \cdot V_A \cdot (1 - 0.5 \cdot (f_s + f_d))$, where f_s and f_d are the inbreeding coefficients of the sire and dam, respectively (Villanueva *et al.*, 1996). Because in our simulations there are no reproducing inbred individuals and V_A equals h^2 , the equation for the variance reduces to $\sigma^2 = 0.5 \cdot h^2$ in our case.

Before mating, the mean phenotypic value of each brother–sister pair was calculated. Brother–sister pairs with high mean phenotypic values (the upper 80%) were randomized until all mating pairs were unrelated. On the other hand, brother–sister pairs having low mean phenotypic values (the lower 20%) mated with each other. Thus, 80% of all matings in the F1 generation were among unrelated individuals, and 20% were among full sibs. To generate the F2 generation, each pair again produced one female and one male offspring as described before (generation F1). Additionally, for

offspring of brother–sister pairs, we added an effect of inbreeding equal to $b \cdot f$, where b is the slope of a regression of phenotype on f . Data were simulated for $h^2 = 0, 0.25, 0.5$ and 0.75 and for $b = -1, 0$ and $+1$. Because brother–sister pairs have inbred offspring (coefficient of inbreeding $f = 0.25$), and given the trait is heritable (i.e. when $h^2 > 0$), we expect offspring of brother–sister pairs to have lower phenotypic values than the offspring of unrelated couples, also in the absence of a direct effect of inbreeding (i.e. when $b = 0$).

Simulated data were analysed using a linear regression of phenotype on inbreeding coefficient using individuals of the F2 generation, which were the result of phenotype-associated inbreeding. For comparison, data were analysed with an animal model using individuals of all three generations to estimate the heritability. Because we were only interested in inbreeding effects in the last generation (F2), we fitted an interaction term between the inbreeding coefficient and generation. This allowed us to predict the inbreeding effect for each generation separately, based on the appropriate combinations of parameter estimates. For each combination of h^2 and b , we simulated 100 replicate data sets.

Note that the main purpose of these simulations is to illustrate how phenotype-associated inbreeding may bias estimates of inbreeding depression and how this bias can be accounted for within a quantitative genetic framework. The specific mechanism to introduce phenotype-associated inbreeding employed here is unlikely to be realistic, and the amounts of inbreeding, inbreeding depression and phenotype-associated inbreeding are likely to be larger than what can reasonably be expected in nature.

Study system and data set

We tested for phenotype-associated inbreeding and its effect in a natural population of white-throated dippers (*Cinclus c. aquaticus*), a medium-sized passerine (in our study population, males are 62.5 ± 3.6 g and females 53.7 ± 4.1 g; mean \pm SD) that is widely distributed across Europe. It lives along streams and rivers and mainly feeds on aquatic invertebrates. After an incubation period of 16–17 days, first clutches hatch between the middle of March and the beginning of May (brood size at ringing: 4.4 ± 1.1 nestlings). About 35% of all offspring are from second clutches (Hegelbach, 2013), which hatch between the end of April and the beginning of June and contain on average 3.7 ± 1.1 nestlings at the time of ringing. Both parents provide food to the offspring, which fledged 21–24 days after hatching.

Since 1987, dippers have been monitored intensively at eleven rivers spanning an area of approximately 400 km² in the proximity of Zurich, Switzerland. Here, we used data from the K  snacht (river length: 6.5 km),

Sihl (25.5 km) and Wehrenbach (5.5 km) rivers. In these rivers, more than 99% of all broods could be accessed, and virtually all breeding individuals were known and identifiable on the basis of a unique combination of one metal and two colour rings (between 1996 and 2013, only 0.1% and 0.5% of ringed nestlings had an unknown mother or father, respectively).

Behavioural observations during the mating and breeding season allowed determining parentage of each brood. Because dippers have a very low rate of extra-pair paternity (2% according to Øigarden *et al.*, 2010; < 1% according to our own unpublished data), we reconstructed the pedigree using behavioural observations only. We calculated Wright's inbreeding coefficient (f) for all individuals since 1987 using the software PEDIGREE VIEWER (available at <http://www.personal.une.edu.au/~bkinghor/pedigree.htm>). Mean and maximum pedigree depths were 4.5 and 15 generations, respectively. Because founders and immigrants by definition have unknown parents and are therefore assigned an (uninformative) inbreeding coefficient of zero, analyses on inbreeding effects are restricted to individuals that have hatched in the study area. We excluded individuals that hatched before 1996 because monitoring was still incomplete in the first years of the study and levels of inbreeding might thus be underestimated. The mean inbreeding coefficient of individuals from the cohorts 1996 to 2013 was 0.026 ± 0.063 (mean \pm SD; max = 0.5, resulting from multiple subsequent close inbreeding events).

Individuals that were ringed as nestlings and stayed in the study area were recaptured from two months of age onwards (by which time they are fully grown) to obtain a measure of wing length (to the nearest 0.5 mm). Adults without rings (i.e. immigrants) were captured, banded and measured usually before the start of the breeding season, but at the latest before the ringing of their own offspring. Since 2008, the state of the primary feathers was scored as worn or not worn, with worn feathers being shorter (difference \pm SE based on a linear mixed-effect model: -0.82 ± 0.18 mm). Sex of fully grown individuals can easily be inferred from body size measurements. Since 2001, a small blood sample is taken to verify phenotypic sex by amplifying the CHD-W and CHD-Z genes using modified versions of the P2 and P8 primers (Griffiths *et al.*, 1998; Hoeck *et al.*, 2009).

In a first step, we confined our analyses to individuals with known feather state, resulting in a data set comprising 288 measurements of 192 individuals of the six cohorts that hatched from 2008 to 2013. We subsequently replicated the analyses with data for birds that hatched between 1996 and 2007, for which feather state was unknown. To make them more comparable to the six-year period 2008–2013, we divided these earlier cohorts into two periods of six years each (474 measurements of 251 individuals from the cohorts 1996

to 2001 and 401 measurements of 229 individuals from the cohorts 2002 to 2007). This allowed us to test how patterns of inbreeding and its effect vary over time, and the generality of our predictions regarding the potential biased estimates of inbreeding effects. As in hindsight the exclusion of feather state as a fixed effect had only negligible effects on estimates of the other covariates (including inbreeding coefficient), we here present results of models without this fixed effect for all the three periods.

Statistical analyses

First, we tested whether inbreeding occurred randomly with respect to wing length by correlating the phenotypic values of parents (mid-parent values) with their coefficient of kinship (i.e. the inbreeding coefficient of their offspring) using a Spearman rank correlation. Furthermore, we report the slope of a linear regression of residual mid-parent wing length on the coefficient of kinship. We considered only parents of those individuals that were used to estimate inbreeding depression later on. We did this separately for the three periods mentioned above, each containing six cohorts. Phenotypic values that were used for these correlations were residuals taken from a regression of wing length (first measure of each individual; in 96.3% of cases taken in its first year of life, 3.7% in the second year) on inbreeding coefficient and natal population, separately for females and males and the three different periods. Individuals with no information for these covariates, that is immigrants, were assigned the mean inbreeding coefficient of breeding individuals with known origin and the value 'unknown' as their natal population, respectively.

Subsequently, we estimated the effect of inbreeding on wing length, again separately for the three time periods. For this, we used offspring of the parents used in the previous analysis, measured when they were fully grown. We first did this using the standard method of fitting a linear mixed-effect model, including sex, and natal population (river) as fixed effects and the inbreeding coefficient as a covariate. Because in many species feathers become longer with age (e.g. Alatalo *et al.*, 1984), we also fitted the age at measurement (in years) as a covariate. Cohort (i.e. year of birth) and individual's identity (ID) were included as random effects to account for random environmental variability among years and for multiple measurements per individual, respectively. In this model, the random individual effect captures the variance among individuals due to both permanent environment and (additive and nonadditive) genetic effects. Importantly, however, it does not account for the genetic covariance among relatives.

In a final step, we extended the mixed model outlined above to an animal model (Kruuk, 2004; Wilson

et al., 2010) by fitting a random additive genetic effect. This animal effect estimates the variance in wing length that is due to additive genetic effects, using information on the relatedness and resemblance in wing length among all individuals in the pedigree. Not only does this allow for the estimation of the narrow-sense heritability (h^2 ; the proportion of phenotypic variance explained by additive genetic effects) of wing length, but most importantly in the present context, it enables us to separate additive genetic effects from the effect of inbreeding.

Statistical analyses were performed using R version 3.0.2 (R Development Core Team, 2013), except for the mixed-model analyses, which were fitted using restricted maximum likelihood (REML) in ASREML version 3.0 (Gilmour *et al.*, 2009). Statistical significance of fixed and random effects was assessed using conditional Wald F-tests and likelihood ratio tests, respectively.

Results

Derivation of expected bias

After accounting for additional fixed effects (e.g. sex or natal population) or random effects (e.g. year of birth), we can define the (residual) phenotype of offspring i (y_i) as

$$y_i = \mu + a_i + b \cdot f_i + e_i, \quad (1)$$

where μ is the population mean, a_i is the additive genetic effect (i.e. breeding value) of offspring i , b is the (true) effect of its inbreeding coefficient (f_i) on the trait of interest (i.e. the amount of inbreeding depression), and e_i is a residual environmental term.

Inbreeding depression is typically estimated as the slope of a regression of offspring phenotype on offspring inbreeding coefficient, so

$$\hat{b} = \frac{\sigma(f_i, y_i)}{\sigma^2(f_i)} \quad (2)$$

Substituting eqn 1 for y_i , we get

$$\hat{b} = \frac{\sigma(f_i, a_i + b \cdot f_i + e_i)}{\sigma^2(f_i)}$$

which, assuming $\sigma(f_i, e_i) = 0$, reduces to

$$\hat{b} = b + \frac{\sigma(f_i, a_i)}{\sigma^2(f_i)}. \quad (3)$$

From this, it follows that the slope of a regression of phenotype on inbreeding coefficient (\hat{b}) will provide a biased estimate of inbreeding depression (b) if a linear relationship exists between an individual's inbreeding coefficient and its breeding value for the trait of

interest. Because f_i equals the coefficient of kinship of the parents of i ($\theta_{p,i}$), and a_i is equal to the mean breeding value of the parents of i ($\bar{a}_{p,i}$), eqn 3 can also be written as

$$\hat{b} = b + \frac{\sigma(\theta_{p,i}, \bar{a}_{p,i})}{\sigma^2(\theta_{p,i})}. \quad (4)$$

This again shows that whenever individuals that mate with relatives are genetically different from those that do not, estimates of inbreeding depression will be biased.

Ideally, we would like to be able to express this bias as a function of the covariance between mean parental phenotype and kinship, which, unlike its additive genetic counterpart, can be readily observed. Doing this requires knowledge of the relationship between mean parental breeding value and mean parental phenotype. This relationship is a function of the relationship between breeding value and phenotype (that is of the heritability), and of how nonrandom inbreeding has arisen. For example, the association between kinship and mean parental breeding values may be zero despite a strong phenotypic association if it is solely mediated by a third environmental variable (e.g. habitat quality), with individuals in poor habitats being in worse condition and being more likely to mate with a relative. Such a scenario might also violate the previously made assumption of $\sigma(f_i, e_i) = 0$. However, although such kind of associations due to nonrandom gene flow should not be neglected (see also Edelaar & Bolnick, 2012), they are particularly difficult to measure in empirical settings, among others due to the difficulties in determining, for example, habitat quality.

To complicate matters further, phenotype-associated inbreeding will generate correlations among parental breeding values and among parental phenotypes (i.e. assortative mating), which under some circumstances may also affect the relationship between mean parental breeding and phenotypic values (Appendix S1) (Falconer & Mackay, 1996). Finally, in the above we assume an absence of any nongenetic sources of resemblance among parents and offspring. Although for these reasons a general derivation of the expected bias as a function of the relationship between mean parental phenotype and parental kinship is beyond the scope of this study, in the supplementary material we derive it for the specific scenario of phenotype-associated inbreeding implemented in the simulations presented below (Appendix S2).

Despite the various complications, from the above it follows that the slope of the regression of phenotype on inbreeding coefficient is expected to be biased whenever there is a relationship between the trait of interest and the probability of inbreeding (i.e. $\sigma(\theta_{p,i}, \bar{y}_{p,i}) > 0$), and when this relationship has a heritable component

($\sigma(\theta_{p,i}, \bar{a}_{p,i}) > 0$). The latter is likely to be true whenever $h^2 > 0$ (Appendix S1). When these conditions are fulfilled and the relationship is positive, that is if parents with a high mean breeding value for the trait of interest are more closely related, the bias is positive (i.e. negative effects of inbreeding become less negative and positive effects become more positive). On the other hand, if the relationship is negative because parents with a low mean breeding value are more closely related, the bias will be negative, resulting in negative effects becoming more negative and positive effects becoming less positive or even negative.

Simulations

The above is confirmed by a direct comparison of the estimates of the slope of phenotype on inbreeding coefficient from a linear model to those from a quantitative genetic animal model, when both are applied to simulated data. As our simulations introduced a negative association between parental phenotype and kinship, negative effects are estimated to be even more negative and positive effects are estimated to be less positive (or even negative) in a linear model, with the size of this bias increasing with increasing heritability (Fig. 1). Importantly, even in the absence of any inbreeding effects, the linear model provides a negative estimate of inbreeding on phenotype whenever the trait is heritable. In stark contrast, the animal model provides estimates of both heritability (not shown) and inbreeding effects (Fig. 1) that on average do not deviate from those simulated.

As discussed above, predicting the amount of bias on the basis of the observed relationship between mean parental phenotype and kinship ($\sigma(\theta_{p,i}, \bar{y}_{p,i})/\sigma^2(\theta_{p,i})$) is not straightforward. However, from our simulations, in combination with eqn 4, it follows that the difference between the inbreeding effect from a linear model and an animal model can provide us with an estimate of $\sigma(\theta_{p,i}, \bar{a}_{p,i})/\sigma^2(\theta_{p,i})$, that is the magnitude of the bias. Dividing this by the observed slope of the regression of mean parental phenotype on parental kinship $\sigma(\theta_{p,i}, \bar{y}_{p,i})/\sigma^2(\theta_{p,i})$, we get $\sigma(\theta_{p,i}, \bar{a}_{p,i})/\sigma(\theta_{p,i}, \bar{y}_{p,i})$, which provides an estimate of the proportion of the phenotypic covariance between a trait and parental kinship that is passed on to the next generation.

Empirical estimation of bias

To obtain an empirical estimate of the bias in estimates of inbreeding effects on wing length in dippers, we first restricted ourselves to the cohorts 2008–2013 (see Materials and methods). During this period, parents were more related to each other when they had shorter wings (Spearman's $\rho = -0.22$, $P = 0.002$, slope and SE of regression of parental wing length on kinship: -2.47 ± 1.19 , $P = 0.04$). From the perspective of the

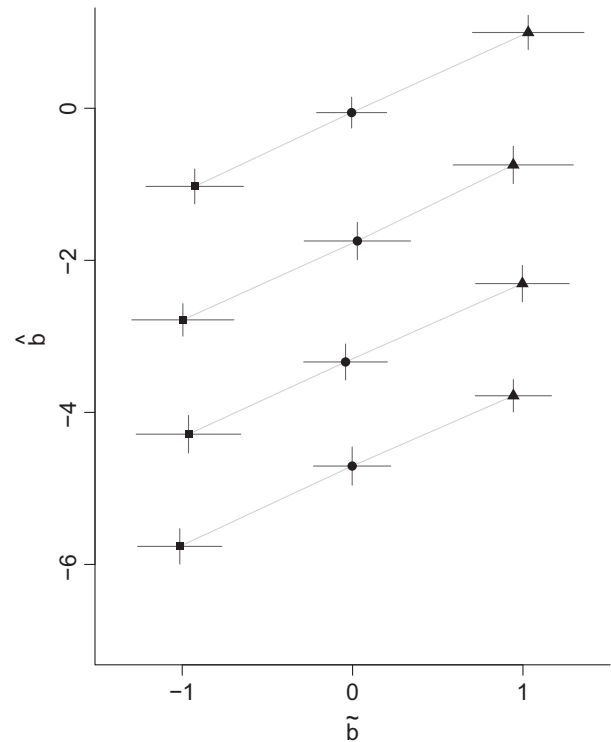


Fig. 1 Inbreeding effects in a simulated population with phenotype-associated inbreeding (full-sib mating in 20% of brother–sister pairs with the lowest mid-parent phenotypic values, and no inbreeding in the remaining 80% of the population). See text for more details. Estimates of the effect of the inbreeding coefficient on the phenotype (mean \pm SD of 100 replicates) from a quantitative genetic animal model (\tilde{b}) are compared with estimates from a linear model (b). Results from simulations with the same simulated heritability are connected through lines ($h^2 = 0, 0.25, 0.5$ and 0.75 , from top to bottom). Different inbreeding effects (b) are depicted as squares (negative effect: $b = -1$, left), circles (no effect: $b = 0$, centre) and triangles (positive effect: $b = +1$, right). Whereas results from the animal model analysis were unbiased, the linear model analysis returned increasingly biased estimates with increasing heritability.

offspring, inbred offspring had parents with shorter wings than outbred offspring.

Without accounting for additive genetic effects (Table 1a), we found that wing length is highly sexually dimorphic, with males having 7.3 mm (SE: 0.22 mm; $F_{1,196.7} = 1056.54$, $P < 0.001$) longer wings than females. Furthermore, wing length increased slightly but significantly with age (0.35 ± 0.09 mm, $F_{1,171.6} = 15.79$, $P < 0.001$). Individuals born in different rivers of the study area did not differ in wing length ($F_{2,182.0} = 1.76$, $P = 0.18$). Most importantly, for our current purposes, wing length decreased significantly with increasing inbreeding coefficient (-5.13 ± 1.68 mm, $F_{1,173.4} = 11.98$, $P = 0.003$), that is, inbred birds had shorter wings. Inclusion of the state of the feathers pro-

duced a very similar estimate of -4.98 ± 1.65 mm. The variance component for individual identity, combining permanent environment and genetic effects, was 1.57 ± 0.27 ($\chi^2 = 58.7$, $P < 0.001$), accounting for $59.4 \pm 6.4\%$ of the phenotypic variance in wing length after accounting for the variance explained by the fixed effects. Variation in wing length among cohorts was negligible (0.024 ± 0.065 , $\chi^2 = 0.19$, $P = 0.33$).

Expanding the above-described mixed model to an animal model to account for additive genetic resemblance among parents and offspring (Table 1b) yielded estimates of the additive genetic variance of 1.59 ± 0.48 ($\chi^2 = 25.3$, $P < 0.001$), providing an estimate of heritability for wing length of $61.0 \pm 13.1\%$. In line with this result, the variance due to individual identity (here only describing the permanent environ-

ment) dropped to 0.11 ± 0.31 , explaining another 3.7% of the phenotypic variance ($\chi^2 = 0.13$, $P = 0.36$). Whereas estimates for the effects of sex, age and natal river were close to those of the mixed model without an additive genetic animal effect, the estimate for the effect of the inbreeding coefficient was now only -3.45 ± 1.96 mm ($F_{1,163.1} = 3.12$, $P = 0.08$), corresponding to a decline of 33% (Fig. 2). Inclusion of the parameter describing the state of the feathers produced an estimate of -3.34 ± 1.93 mm. From this decline ([estimate without animal effect] – [estimate with animal effect] = -1.68) and the observed relationship between mean parental wing length and kinship (-2.47 ± 1.19), we obtain $\sigma(\theta_{p,i}, \bar{a}_{p,i})/\sigma(\theta_{p,i}, \bar{y}_{p,i}) = 0.68$, which is slightly larger than the heritability of 0.61 .

Table 1 Analysis of variance in wing length in white-throated dippers. Estimates of fixed and random effects are given from (a) standard linear mixed-effect models and (b) from animal models. Inbreeding coefficient (f), sex, age (in years) and river were included as fixed effects, and individual identity (ID) and cohort (year) as random effects. Animal models furthermore estimated additive genetic variance (animal). Data were analysed separately for the cohorts 1996–2001, 2002–2007 and 2008–2013.

(a)	1996–2001				2002–2007				2008–2013			
	Estimate	Test statistic	P-value		Estimate	Test statistic	P-value		Estimate	Test statistic	P-value	
Fixed effect	\pm SE				\pm SE				\pm SE			
Intercept	92.92 ± 0.23				92.19 ± 0.23				91.65 ± 0.25			
f	-2.48 ± 1.43	$F_{1,255.9} = 3.00$	0.087		2.59 ± 1.96	$F_{1,224.2} = 1.75$	0.19		-5.13 ± 1.68	$F_{1,173.4} = 11.98$	0.003	
Sex	-7.75 ± 0.19	$F_{1,239.5} = 1640.39$	< 0.001		-7.82 ± 0.20	$F_{1,221.5} = 1535.23$	< 0.001		-7.30 ± 0.22	$F_{1,196.7} = 1056.54$	< 0.001	
(female)												
Age	0.13 ± 0.04	$F_{1,334.3} = 11.97$	< 0.001		0.36 ± 0.04	$F_{1,309.9} = 66.00$	< 0.001		0.35 ± 0.09	$F_{1,171.6} = 15.79$	< 0.001	
River		$F_{2,238.0} = 4.88$	0.009			$F_{2,216.1} = 0.91$	0.41			$F_{2,182.0} = 1.76$	0.18	
Random effect	Variance	Test statistic	P-value		Variance	Test statistic	P-value		Variance	Test statistic	P-value	
	\pm SE				\pm SE				\pm SE			
Individual (V_{ID})	1.47 ± 0.20	$\chi^2 = 125.1$	< 0.001		1.27 ± 0.21	$\chi^2 = 74.0$	< 0.001		1.57 ± 0.27	$\chi^2 = 58.7$	< 0.001	
Cohort (V_{YEAR})	0.04 ± 0.06	$\chi^2 = 0.7$	0.20		0.12 ± 0.11	$\chi^2 = 4.8$	0.014		0.02 ± 0.06	$\chi^2 = 0.19$	0.33	
Residual (V_R)	0.97 ± 0.09				1.26 ± 0.13				1.05 ± 0.15			
(b)	1996–2001				2002–2007				2008–2013			
	Estimate	Test statistic	P-value		Estimate	Test statistic	P-value		Estimate	Test statistic	P-value	
Fixed effect	\pm SE				\pm SE				\pm SE			
Intercept	93.27 ± 0.42				92.11 ± 0.28				91.54 ± 0.42			
f	-3.10 ± 1.84	$F_{1,168.8} = 2.85$	0.10		2.66 ± 2.24	$F_{1,205.7} = 1.42$	0.24		-3.45 ± 1.96	$F_{1,163.1} = 3.12$	0.08	
Sex	-7.69 ± 0.18	$F_{1,206.6} = 1917.02$	< 0.001		-7.79 ± 0.18	$F_{1,207.7} = 1780.57$	< 0.001		-7.26 ± 0.21	$F_{1,189.7} = 1249.88$	< 0.001	
(female)												
Age	0.15 ± 0.04	$F_{1,352.0} = 14.57$	< 0.001		0.34 ± 0.04	$F_{1,325.0} = 61.00$	< 0.001		0.33 ± 0.08	$F_{1,188.4} = 15.87$	< 0.001	
River		$F_{2,47.2} = 3.11$	0.054			$F_{2,92.4} = 0.08$	0.93			$F_{2,69.3} = 0.84$	0.44	
Random effect	Variance	Test statistic	P-value		Variance	Test statistic	P-value		Variance	Test statistic	P-value	
	\pm SE				\pm SE				\pm SE			
Animal (V_A)	1.23 ± 0.43	$\chi^2 = 20.1$	< 0.001		1.22 ± 0.39	$\chi^2 = 19.5$	< 0.001		1.74 ± 0.48	$\chi^2 = 25.3$	< 0.001	
Individual (V_{ID})	0.37 ± 0.28	$\chi^2 = 1.5$	0.11		0.16 ± 0.27	$\chi^2 = 0.4$	0.27		0.11 ± 0.30	$\chi^2 = 0.1$	0.36	
Cohort (V_{YEAR})	0.06 ± 0.07	$\chi^2 = 2.1$	0.07		0.11 ± 0.10	$\chi^2 = 5.3$	0.01		$1 \times 10^{-7} \pm 1 \times 10^{-8}$	$\chi^2 = 0$	0.50	
Residual (V_R)	0.99 ± 0.09				1.26 ± 0.13				1.01 ± 0.14			

In a next step, we explored the patterns in the previous cohorts. Contrary to the period 2008–2013 (see above), kinship coefficients increased slightly albeit not significantly with increasing (mid-parent) wing length in the first period (1996–2001) (Spearman's $\rho = 0.12$, $P = 0.07$; slope and SE of regression of parental wing length on kinship: 0.89 ± 0.83 , $P = 0.29$). Thus, between 1996 and 2001 inbred individuals tended to have parents with longer wings compared to outbred individuals. In the second period (2002–2007), inbreeding was independent of parental wing length (Spearman's $\rho = -0.03$, $P = 0.70$; slope and SE of regression of (residual) parental wing length on kinship: 0.65 ± 1.29 , $P = 0.62$).

As before, we analysed variation in wing length using standard mixed-effect models, and animal models accounting for additive genetic effects. Variance component estimates for the permanent environment, cohort and additive genetic effects and estimates of the fixed effects are given in Table 1. Heritability was $46.5 \pm 13\%$ in the first (1996–2001) and $44.5 \pm 12\%$ in the second period (2002–2007). In line with the differences in the associations between wing length and kinship coefficients in parents between the three periods, accounting for additive genetic effects changed estimates of inbreed-

ing effects in different ways. In the first period, when related parents had slightly longer wings, the point estimate for the effect of inbreeding from the animal model was slightly larger than from the standard mixed-effect model (-3.10 ± 1.8 mm, $F_{1,168.8} = 2.85$, $P = 0.10$ vs. -2.48 ± 1.4 mm, $F_{1,255.9} = 3.00$, $P = 0.09$, Fig. 2). In the second period, when we detected no association between wing length and kinship coefficients in parents, the effect of the inbreeding coefficient on wing length changed even less between the two types of models (animal model: $+2.66 \pm 2.24$ mm, $F_{1,205.7} = 1.42$, $P = 0.24$; standard mixed model: $+2.59 \pm 1.96$, $F_{1,224.2} = 1.75$, $P = 0.19$) and the point estimate was positive (Fig. 2).

Including all three periods in an animal model, the overall effect of inbreeding on wing length was negative (-2.25 ± 1.11 , $F_{1,577.6} = 4.11$, $P = 0.045$). Additionally, including period as a fixed effect showed that mean wing length differed only slightly between the three periods ($F_{2,31.7} = 2.73$, $P = 0.08$). However, adding the interaction between inbreeding coefficient and the period as an additional term in the animal model showed that inbreeding effects on wing length differed significantly between the three different periods ($F_{2,502.1} = 5.92$, $P = 0.003$). Similarly, phenotype-associated inbreeding differed between the three periods, as the correlation between wing length and parental kinship was significantly negative in one period and positive in another one.

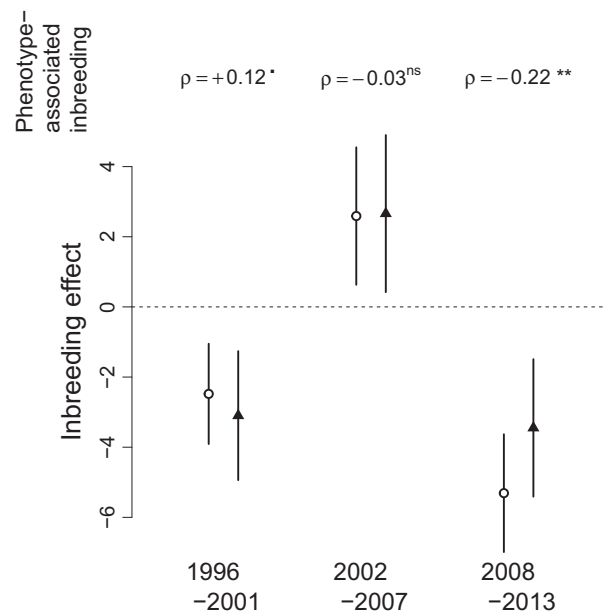


Fig. 2 Effect of the inbreeding coefficient f on wing length (mean \pm SE), based on standard linear mixed-effect models (open circles) and animal models (closed triangles). If additive genetic effects were not accounted for, inbreeding effects were slightly underestimated when the correlation between kinship coefficient and wing length in parents was positive (cohorts 1996–2001) and substantially overestimated when the correlation was negative (cohorts 2008–2013). When there was no correlation, the estimate was unbiased (middle; cohorts 2002–2007).

Discussion

Here we used a combination of simulations and long-term data on white-throated dippers to investigate whether phenotype-associated inbreeding biases estimates of inbreeding depression. We found that biases can be substantial, especially if traits are highly heritable, but that by estimating inbreeding effects in an animal model, we are able to reduce these biases.

In our study population, dispersal behaviour is correlated with wing length, with individuals that disperse to another river having on average longer wings ($+0.33 \pm 0.12$ mm, $t = 2.62$, $P = 0.009$, unpublished data) than individuals that stay in their natal river (philopatric individuals). Similar size-dependent dispersal behaviour has been shown in a range of other species (Paradis *et al.*, 1998; Skjelseth *et al.*, 2007; Dawideit *et al.*, 2009) (but see Chaput-Bardy *et al.*, 2010). This, combined with typically higher probabilities of inbreeding in philopatric individuals (Szulkin & Sheldon, 2008), has the potential to generate a negative association between wing length and the probability of inbreeding.

Indeed, during the last six years of the study (2008–2013), shorter-winged parents were more likely to mate with a relative. Similarly, in song sparrows, males with shorter tarsi paired with more closely related mates (Reid *et al.*, 2008). Interestingly, however,

the relationship between wing length and the relatedness of parents varied over time in our dipper populations. In the first period (1996–2001), there was a tendency that longer-winged individuals were more likely to mate with a relative. In the second period (2002–2007), inbreeding occurred randomly with respect to wing length. The reasons for this heterogeneous pattern are unknown but might involve differences in mate availability between the different periods, or variation in patterns of mate choice with respect to relatedness, wing length or other traits. Whatever the reasons for this temporal variation in phenotype-associated inbreeding, it allowed us to demonstrate that it can cause variation in the bias of estimates of inbreeding effects.

Our simulation results confirm that ignoring additive genetic variation in traits associated with the occurrence of inbreeding leads to biased estimates of inbreeding effects. In line with this, phenotype-associated inbreeding in white-throated dippers caused biases in the estimates of inbreeding effects on wing length: inbreeding effects were slightly underestimated if inbred individuals had slightly longer-winged parents, unbiased if inbred individuals had parents of average wing length, and substantially overestimated if inbred individuals had significantly shorter-winged parents. Thus, variation in phenotype-associated inbreeding resulted in quantitatively different biases in the different time periods. Whereas Lynch & Walsh (1998, p. 272) were primarily concerned with the overestimation of inbreeding depression in fitness traits that results when parents that inbreed are also genetically low on the fitness scale, our results suggest that inbreeding depression can not only be overestimated, but also be underestimated. This occurs when parents that are genetically high on the fitness (or other phenotype) scale are more likely to inbreed, as has been demonstrated in great tits (Van Noordwijk & Scharloo, 1981).

Our results suggest that the intensity of both phenotype-associated inbreeding and inbreeding effect varied over time. This remained true after accounting for the biases that phenotype-associated inbreeding introduces. Differences in the magnitude of inbreeding depression can be caused by environmental variation (Cheptou & Donohue, 2011), for example with stronger inbreeding depression under more adverse conditions and less inbreeding depression under more benign conditions, as shown in Darwin's finches (*Geospiza scandens*) and song sparrows (Keller *et al.*, 2002; Marr *et al.*, 2006). In addition, changes in the sign of inbreeding effects may be mediated by genetic changes over time (Curik *et al.*, 2001).

We have shown phenotype-associated inbreeding in this particular population of white-throated dipper, but how general is this phenomenon? In principle, any mechanism that leads to a correlation between an indi-

vidual's phenotype and its kinship coefficient with its mate will lead to this phenomenon. As discussed in detail by Reid *et al.* (2008), such correlations can arise both under random mating, for example when reproductive success varies with phenotype, and when mate choice is nonrandom, for example if individuals with a particular phenotype either prefer or are forced to mate with kin more often than expected by chance. In addition, phenotype-dependent dispersal may generate phenotype-associated inbreeding under random mating. Although in many evolutionary genetic models dispersal and gene flow are assumed to be random and thus phenotype independent (see Lenormand, 2002; Räsänen & Hendry, 2008), this assumption may be violated in natural populations (Edelaar & Bolnick, 2012). For example, dispersing and philopatric individuals may differ in their morphology (e.g. Paradis *et al.*, 1998; Skjelseth *et al.*, 2007; Dawideit *et al.*, 2009), in their personality (e.g. Cote *et al.*, 2011) or in fitness-related traits (e.g. Friedenberg, 2003; Innocent *et al.*, 2010). Furthermore, immigrants can differ in traits not directly related to dispersal, but which differ between the population of origin and the receiving population, for example due to local adaptation (e.g. Postma & Van Noordwijk, 2005).

Hence, conditions for phenotype-associated inbreeding are likely to be fulfilled in many populations, in particular in populations living in spatially fragmented environments. Studies of inbreeding depression have to be aware of the potential biases this may introduce. However, the size of this bias is related to the heritability of the trait. Although most traits do show additive genetic variance, heritability is usually lower in fitness-related traits than in morphological traits (Mousseau & Roff, 1987; Postma, 2014), suggesting that a potential bias will be smaller in the former.

A straightforward approach to reducing the potential bias in estimates of inbreeding depression caused by phenotype-associated inbreeding is to simultaneously account for the inbreeding coefficient and additive genetic effects (see also Uimari & Kennedy, 1990). As we have shown here, this is possible within a quantitative genetic animal model framework. Because inbreeding coefficients in individual long-term studies are generally calculated using pedigree data, this requires no additional data. Indeed, whereas the importance of including additive genetic effects when estimating the effect of inbreeding has so far obtained less attention, the importance of including inbreeding coefficients as an additional covariate in the animal model to obtain unbiased estimates of additive genetic variance and heritability has been emphasized repeatedly (Hoeschele & Van Raden, 1991; De Boer & Van Arendonk, 1992; reviewed in Wolak & Keller, 2014). Moreover, excluding inbreeding coefficients can systematically inflate estimates of heritability if inbreeding coefficients of parents and offspring are correlated (Reid *et al.*, 2006).

Such correlations can arise even under random mating in fragmented and variable environments where relatively isolated subpopulations are connected through occasional long-distance dispersers (Reid *et al.*, 2006). However, in our data set the relative change in estimates of heritability was only marginal when excluding inbreeding coefficient (estimates of h^2 for the three periods excluding inbreeding coefficient were 0.46 ± 0.13 , 0.45 ± 0.12 and 0.64 ± 0.13 ; estimates of h^2 for the three periods with inbreeding coefficient were 0.47 ± 0.13 , 0.45 ± 0.12 and 0.61 ± 0.13).

In summary, we have shown that phenotype-associated inbreeding may bias estimates of the effect of inbreeding, in simulated data as well as in a long-term study population of white-throated dippers. However, patterns varied over time, with inbred individuals having on average longer-winged parents in some cohorts, but shorter-winged parents in other cohorts. When we did not account for additive genetic effects, these patterns of phenotype-associated inbreeding resulted in an under- or overestimation of inbreeding effects. Because phenotype-associated inbreeding, in particular due to phenotype-dependent dispersal in spatially fragmented environments, is likely to be reasonably widespread, it should be accounted for in future studies of inbreeding depression.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Relationships among phenotypes, breeding values and environmental deviations of a simulated population. See Appendix S2 for more information.

Appendix S2 Illustration of the relationship between mean parental breeding values and phenotypes.

Figure S1 Relationships among phenotypes, breeding values and environmental deviations of a simulated population. See Appendix S2 for more information.

Table S1 Estimates of all relevant variances and correlations for the inbreeding ($\theta = 0.25$) and outbreeding ($\theta = 0$) parts of a simulated population. For more details, see Appendix S2.

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